

jc759 U.S. PTO  
03/28/00

03-30-60

A

Practitioner's Docket No. 49674

PATENT

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.

jc544 U.S. PTO  
09/537858  
03/28/00

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 20231

### NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Paul PROOST, Sofie STRUYF and Jo VAN DAMME

**WARNING:** 37 C.F.R. § 1.41(a)(1) points out:

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

#### CERTIFICATION UNDER 37 C.F.R. § 1.10\*

(Express Mail label number is mandatory.)

(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date March 28, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number TB553892394US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Peter F. Corless

(type or print name of person mailing paper)

Signature of person mailing paper

**WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

**\*WARNING:** Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

(New Application Transmittal [4-1]—page 1 of 11)

Cognitive Function		Mood		Quality of Life		Social Function		Health Status	
Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention	Pre-Intervention	Post-Intervention
10.5	11.2	1.5	1.8	2.5	2.8	3.5	3.8	4.5	4.8
11.5	12.2	1.8	2.1	2.8	3.1	3.8	4.1	4.8	5.1
12.5	13.2	2.1	2.4	3.1	3.4	4.1	4.4	5.1	5.4
13.5	14.2	2.4	2.7	3.4	3.7	4.4	4.7	5.4	5.7
14.5	15.2	2.7	3.0	3.7	4.0	4.7	5.0	5.7	6.0
15.5	16.2	3.0	3.3	4.0	4.3	5.0	5.3	6.0	6.3
16.5	17.2	3.3	3.6	4.3	4.6	5.3	5.6	6.3	6.6
17.5	18.2	3.6	3.9	4.6	4.9	5.6	5.9	6.6	6.9
18.5	19.2	3.9	4.2	4.9	5.2	5.9	6.2	6.9	7.2
19.5	20.2	4.2	4.5	5.2	5.5	6.2	6.5	7.2	7.5
20.5	21.2	4.5	4.8	5.5	5.8	6.5	6.8	7.5	7.8
21.5	22.2	4.8	5.1	5.8	6.1	6.8	7.1	7.8	8.1
22.5	23.2	5.1	5.4	6.1	6.4	7.1	7.4	8.1	8.4
23.5	24.2	5.4	5.7	6.4	6.7	7.4	7.7	8.4	8.7
24.5	25.2	5.7	6.0	6.7	7.0	7.7	8.0	8.7	9.0
25.5	26.2	6.0	6.3	7.0	7.3	8.0	8.3	9.0	9.3
26.5	27.2	6.3	6.6	7.3	7.6	8.3	8.6	9.3	9.6
27.5	28.2	6.6	6.9	7.6	7.9	8.6	8.9	9.6	9.9
28.5	29.2	6.9	7.2	7.9	8.2	8.9	9.2	9.9	10.2
29.5	30.2	7.2	7.5	8.2	8.5	9.2	9.5	10.2	10.5
30.5	31.2	7.5	7.8	8.5	8.8	9.5	9.8	10.5	10.8
31.5	32.2	7.8	8.1	8.8	9.1	9.8	10.1	10.8	11.1
32.5	33.2	8.1	8.4	9.1	9.4	10.1	10.4	11.1	11.4
33.5	34.2	8.4	8.7	9.4	9.7	10.4	10.7	11.4	11.7
34.5	35.2	8.7	9.0	9.7	10.0	10.7	11.0	11.7	12.0
35.5	36.2	9.0	9.3	10.0	10.3	11.0	11.3	12.0	12.3
36.5	37.2	9.3	9.6	10.3	10.6	11.3	11.6	12.3	12.6
37.5	38.2	9.6	9.9	10.6	10.9	11.6	11.9	12.6	12.9
38.5	39.2	9.9	10.2	10.9	11.2	11.9	12.2	12.9	13.2
39.5	40.2	10.2	10.5	11.2	11.5	12.2	12.5	13.2	13.5
40.5	41.2	10.5	10.8	11.5	11.8	12.5	12.8	13.5	13.8
41.5	42.2	10.8	11.1	11.8	12.1	12.8	13.1	13.8	14.1
42.5	43.2	11.1	11.4	12.1	12.4	13.1	13.4	14.1	14.4
43.5	44.2	11.4	11.7	12.4	12.7	13.4	13.7	14.4	14.7
44.5	45.2	11.7	12.0	12.7	13.0	13.7	14.0	14.7	15.0
45.5	46.2	12.0	12.3	13.0	13.3	14.0	14.3	15.0	15.3
46.5	47.2	12.3	12.6	13.3	13.6	14.3	14.6	15.3	15.6
47.5	48.2	12.6	12.9	13.6	13.9	14.6	14.9	15.6	15.9
48.5	49.2	12.9							

**This new application is for a(n)**

**(check one applicable item below)**

**Original (nonprovisional)**

- ☐ Design
- ☐ Plant

**WARNING:** Do not use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.

**WARNING:** Do not use this transmittal for the filing of a provisional application.

**NOTE:** If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

- ☐ Divisional.
- ☒ Continuation.
- ☐ Continuation-in-part (C-I-P).

**2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)**

**NOTE:** A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

- (i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or
- (ii) Complete as set forth in § 1.51(b); or
- (iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or
- (iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(f) within the time period set forth in § 1.53(f).

**37 C.F.R. § 1.78(a)(1).**

**NOTE:** If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach **ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.**

**WARNING:** *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

Variable	Mean	SD	Min	Max	Skewness	Kurtosis
Age	38.5	12.5	25	65	-0.1	3.2
Gender	0.5	0.5	0	1	0.0	3.0
Marital Status	0.7	0.4	0	1	0.1	3.1
Education	12.5	2.5	9	16	-0.2	3.3
Income	3500	1500	1000	8000	0.3	3.4
Occupation	1.5	1.0	1	5	0.0	3.0
Health	2.5	1.5	1	5	-0.1	3.2
Stress	3.0	1.0	1	5	0.2	3.5
Depression	1.5	1.0	1	5	-0.1	3.2
Life Satisfaction	3.5	1.0	1	5	-0.2	3.3
Resilience	2.0	1.0	1	5	0.1	3.1
Optimism	3.0	1.0	1	5	-0.1	3.2
Emotional Stability	2.5	1.0	1	5	0.0	3.0
Self-Esteem	3.0	1.0	1	5	-0.1	3.2
Life Satisfaction	3.5	1.0	1	5	-0.2	3.3
Resilience	2.0	1.0	1	5	0.1	3.1
Optimism	3.0	1.0	1	5	-0.1	3.2
Emotional Stability	2.5	1.0	1	5	0.0	3.0
Self-Esteem	3.0	1.0	1	5	-0.1	3.2

- ### 3. Papers Enclosed

- 10 Sheets of drawing

**NOTE:** "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c).

☐ informal

2 Other Pages of Sequence Listing

## ☐ Citations

☐ Declaration of Biological Deposit

☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

Other \_\_\_\_\_

**NOTE:** A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

**Executed by**

(check all applicable boxes)

- ☐ inventor(s).
- ☐ legal representative of inventor(s).  
37 C.F.R. §§ 1.42 or 1.43.
- ☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.
  - ☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☒ Not Enclosed.

**NOTE:** Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

- ☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

- ☐ Showing that the filing is authorized.  
(not required unless called into question. 37 C.F.R. § 1.41(d))

Parameter	Value	Unit
Initial concentration	1.0	g/L
Initial pH	7.0	
Temperature	25	°C
Time	0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, 2097152, 4194304, 8388608, 16777216, 33554432, 67108864, 134217728, 268435456, 536870912, 1073741824, 2147483648, 4294967296, 8589934592, 17179869184, 34359738368, 68719476736, 137438953472, 274877906944, 549755813888, 1099511627776, 2199023255552, 4398046511104, 8796093022208, 17592186044416, 35184372088832, 70368744177664, 140737488355328, 281474976710656, 562949953421312, 1125899906842624, 2251799813685248, 4503599627370496, 9007199254740992, 18014398509481984, 36028797018963968, 72057594037927936, 144115188075855872, 288230376151711744, 576460752303423488, 1152921504606846976, 2305843009213693952, 4611686018427387904, 9223372036854775808, 18446744073709551616, 36893488147419103232, 73786976294838206464, 147573952589676412928, 295147905179352825856, 590295810358705651712, 1180591620717411303424, 2361183241434822606848, 4722366482869645213696, 9444732965739290427392, 18889465931478580854784, 37778931862957161709568, 75557863725914323419136, 151115727451828646838272, 302231454903657293676544, 604462909807314587353088, 1208925819614629174706176, 2417851639229258349412352, 4835703278458516698824704, 9671406556917033397649408, 19342813113834066795298816, 38685626227668133590597632, 77371252455336267181195264, 154742504910672534362390528, 309485009821345068724781056, 618970019642690137449562112, 1237940039285380274899124224, 2475880078570760549798248448, 4951760157141521099596496896, 9903520314283042199192993792, 19807040628566084398385987584, 39614081257132168796771975168, 79228162514264337593543950336, 158456325028528675187087900672, 316912650057057350374175801344, 633825300114114700748351602688, 1267650600228229401496703205376, 2535301200456458802993406410752, 5070602400912917605986812821504, 10141204801825835211973625643008, 20282409603651670423947251286016, 40564819207303340847894502572032, 81129638414606681695789005144064, 162259276829213363391578010288128, 324518553658426726783156020576256, 649037107316853453566312041152512, 1298074214633706907132624082305024, 2596148429267413814265248164610048, 5192296858534827628530496329220096, 10384593717069655257060992658440192, 20769187434139310514121985316880384, 41538374868278621028243970633760768, 83076749736557242056487941267521536, 166153499473114484112975882535043072, 332306998946228968225951765070086144, 664613997892457936451903530140172288, 1329227995784915872903807060280344576, 2658455991569831745807614120560689152, 5316911983139663491615228241121378304, 10633823966279326983230456482242756608, 21267647932558653966460912964485513216, 42535295865117307932921825928971026432, 85070591730234615865843651857942052864, 170141183460469231731687303715884105728, 340282366920938463463374607431768211456, 680564733841876926926749214863536422912, 1361129467683753853853498429727072845824, 2722258935367507707706996859454145691648, 5444517870735015415413993718908291383296, 10889035741470030830827987437816582766592, 21778071482940061661655974875633165533184, 43556142965880123323311949751266331066368, 87112285931760246646623899502532662132736, 174224571863520493293247799005065324265472, 348449143727040986586495598010130648530944, 696898287454081973172991196020261297061888, 1393796574908163946345982392040522594123776, 2787593149816327892691964784081045188247552, 5575186299632655785383929568162090376495104, 11150372599265311570767859136324180752990208, 22300745198530623141535718272648361505980416, 44601490397061246283071436545296723011960832, 89202980794122492566142873090593446023921664, 178405961588244985132285746181186892047843328, 356811923176489970264571492362373784095686656, 713623846352979940529142984724747568191373312, 1427247692705959881058285969449495136382746624, 2854495385411919762116571938898990272765493248, 5708990770823839524233143877797980545530986496, 11417981541647679048466287755595961091061972992, 2283596308329	

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☐ The same.

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ will be submitted.

**NOTE:** An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of \$130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

☐ Non-English

## 8. Assignment

☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

**NOTE:** "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

(New Application Transmittal [4-1]—page 5 of 11)

Figure 1 consists of 12 bar charts, labeled (a) through (l), each representing a different demographic or socio-economic variable. Each chart compares the percentage of respondents in a specific category (represented by the left bar) with the percentage of the total sample in that category (represented by the right bar). The variables are as follows:

- (a) Age:** Categories include 18-24, 25-34, 35-44, 45-54, 55-64, 65-74, and 75+.
- (b) Sex:** Categories are Male and Female.
- (c) Education:** Categories include Less than high school, High school, Some college, Bachelor's, Master's, and Doctorate.
- (d) Employment:** Categories include Full-time, Part-time, Unemployed, and Retired.
- (e) Income:** Categories include Less than \$10,000, \$10,000-\$19,999, \$20,000-\$29,999, \$30,000-\$39,999, \$40,000-\$49,999, \$50,000-\$59,999, \$60,000-\$69,999, \$70,000-\$79,999, \$80,000-\$89,999, \$90,000-\$99,999, and \$100,000+.
- (f) Home ownership:** Categories are Own and Rent.
- (g) Marital status:** Categories include Single, Married, Divorced, and Widowed.
- (h) Religion:** Categories include No religion, Christianity, Islam, Hinduism, Buddhism, and Other.
- (i) Political affiliation:** Categories include Republican, Democrat, Independent, and Other.
- (j) Ethnicity:** Categories include White, Black, Hispanic, Asian, and Other.
- (k) Health status:** Categories include Excellent, Good, Fair, and Poor.
- (l) Disability status:** Categories include No disability and Disability.

Europe	97116863.8	29 September 1997
Country	Appl. No.	Filed
Europe	97122471.2	19 December 1997
Country	Appl. No.	Filed
Europe	98104216.1	10 March 1998
Country	Appl. No.	Filed

☒ will follow.

**NOTE:** This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

**A.  Regular application**

CLAIMS AS FILED				
Number filed		Number Extra	Rate	Basic Fee 37 C.F.R. § 1.16(a) \$760.00
<b>Total</b>				
Claims (37 C.F.R. § 1.16(c))	18	- 20 =	×	\$ 18.00
<b>Independent</b>				
Claims (37 C.F.R. § 1.16(b))	2	- 3 =	×	\$ 78.00
<b>Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))</b>				
			+	\$260.00      260.00

- ☐ Amendment cancelling extra claims is enclosed.
- ☐ Amendment deleting multiple-dependencies is enclosed.
- ☐ Fee for extra claims is not being paid at this time.

**Filing Fee Calculation** **\$ 950.00**

- Filing Fee Calculation \$\_\_\_\_\_

- Filing fee calculation \$\_\_\_\_\_

**11. Small Entity Statement(s)**

- ☐ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

**WARNING:** "Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).

**WARNING:** "Small entity status must not be established when the person or persons signing the . . . statement can unequivocally make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).

(complete the following, if applicable)

- ☐ Status as a small entity was claimed in prior application  
\_\_\_\_\_ / \_\_\_\_\_, filed on \_\_\_\_\_, from which benefit  
is being claimed for this application under:

35 U.S.C. § ☐ 119(e),  
☐ 120,  
☐ 121,  
☐ 365(c),

and which status as a small entity is still proper and desired.

- ☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of A, B or C above)

\$ \_\_\_\_\_

**NOTE:** Any excess of the full fee paid will be refunded if small entity status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

**12. Request for International-Type Search (37 C.F.R. § 1.104(d))**

(complete, if applicable)

- ☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

[illegible]

☒ Not Enclosed

☒ No filing fee is to be paid at this time.

***(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)***

☐ **Enclosed**☐ Filing fee

\$ \_\_\_\_\_

☐ Recording assignment

(\$40.00; 37 C.F.R. § 1.21(h))

(See attached "COVER SHEET FOR  
ASSIGNMENT ACCOMPANYING NEW  
APPLICATION".)

**\$** \_\_\_\_\_

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached

(\$130.00; 37 C.F.R. §§ 1.47 and 1.17(i))

\$ \_\_\_\_\_

☐ For processing an application with a specification in

a non-English language

(\$130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))

\$ \_\_\_\_\_

☐ Processing and retention fee

(\$130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))

\$ \_\_\_\_\_

☐ **Fee for international-type search report**

(\$40.00; 37 C.F.R. § 1.21(e))

\$ \_\_\_\_\_

NOTE: 37 C.F.R. § 1.21(f) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(f) must be paid, within 1 year from notification under § 53(f).

**Total fees enclosed**

\$\_\_\_\_\_

#### 14. Method of Payment of Fees

☐ Check in the amount of \$\_\_\_\_\_

☐ Charge Account No. \_\_\_\_\_ in the amount of  
\$\_\_\_\_\_.

**A duplicate of this transmittal is attached.**

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).



Variable	Mean		SD		t		p	
	Control	Case	Control	Case	Control	Case	Control	Case
Age	24.5	24.5	1.5	1.5	0.0	0.0	1.0	1.0
Gender	10	10	0	0	0.0	0.0	1.0	1.0
Education	12.5	12.5	1.0	1.0	0.0	0.0	1.0	1.0
Occupation	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Marital status	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Religion	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Income	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Health status	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Family size	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Urban/rural	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Employment	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Marriage	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorce	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widow	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Single	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Married	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Widowed	1.5	1.5	1.0	1.0	0.0	0.0	1.0	1.0
Divorced	1.5	1.5	1.0					

**WARNING:** Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.

- ☐ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)
- ☐ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

- ☐ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- ☐ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).
- ☐ 37 C.F.R. § 1.17 (application processing fees)


☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

**NOTE:** 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . ." From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[illegible]

☐ Credit Account No. \_\_\_\_\_

☐ Refund

  
SIGNATURE OF PRACTITIONER

Peter F. Corless  
(type or print name of attorney)  
Dike, Bronstein, Roberts & Cushman, LLP  
130 Water Street  
P.O. Address

**Boston, MA 02109**

☒ **Incorporation by reference of added pages**

*(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)*

- ☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

- ☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added \_\_\_\_\_

- ☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added \_\_\_\_\_

- ☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added \_\_\_\_\_

☐ **Statement Where No Further Pages Added**

*(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)*

- ☐ This transmittal ends with this page.

**ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF  
PRIOR U.S. APPLICATION(S) CLAIMED**

NOTE: See 37 C.F.R. § 1.78.

**17. Relate Back**

**WARNING:** If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:**A. 35 U.S.C. § 119(e)**

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☐ "This application claims the benefit of U.S. Provisional Application(s) No(s).:**APPLICATION NO(S).:****FILING DATE**

_____ / _____	_____ "
_____ / _____	_____ "
_____ / _____	_____ "

[illegible]

☒ "This application is a  
☒ continuation  
☐ continuation-in-part  
☐ divisional

☐ application number 0 / \_\_\_\_\_ filed on \_\_\_\_\_”  
☒ International Application PCT/EP98/06143 filed on  
28 September 1998 and which designated the U.S.”

**NOTE:** The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

*"The Patent and Trademark Office considers the international application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."*

☐ "The nonprovisional application designated above, namely application \_\_\_\_\_ / \_\_\_\_\_, filed \_\_\_\_\_, claims the benefit of U.S. Provisional Application(s) No(s).:

**FILING DATE**

\_\_\_\_\_ / \_\_\_\_\_

\_\_\_\_\_ / \_\_\_\_\_

\_\_\_\_\_ / \_\_\_\_\_

☐ Where more than one reference is made above, please combine all references into one sentence.

**18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application**

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

<b>International</b>	<b>PCT/EP98/06143</b>	<b>September 28, 1998</b>
<b>Country</b>	<b>Appln. no.</b>	<b>Filed on</b>

**The certified copy(ies) has (have)**

- ☐ been filed on \_\_\_\_\_, in prior application 0 / \_\_\_\_\_, which was filed on \_\_\_\_\_.
- ☐ is (are) attached.

**WARNING:** The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).

## 19. Maintenance of Copendency of Prior Application

**NOTE:** The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

- A. ☐ Extension of time in prior application**

**(This item must be completed and the papers filed in the prior application, if the period set in the prior application has run.)**

- ☐ A petition, fee and response extends the term in the pending **prior** application until \_\_\_\_\_
- ☐ A **copy** of the petition filed in prior application is attached.

- B. ☐ Conditional Petition for Extension of Time in Prior Application**

*(complete this item, if previous item not applicable):*

- ☐ A conditional petition for extension of time is being filed in the pending prior application.
- ☐ A **copy** of the conditional petition filed in the prior application is attached.

**20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed**

*(complete applicable item (a), (b) and/or (c) below)*

- (a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are
- ☐ the same.
  - ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

---

*(type name(s) of inventor(s) to be deleted)*

- (b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are
- ☐ the same.
  - ☐ the following additional inventor(s) have been added:

---

*(type name(s) of inventor(s) to be added)*

- (c) The inventorship for all the claims in this application are
- ☐ the same.
  - ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made
    - ☐ is submitted.
    - ☐ will be submitted.

00000000-00000000

Parameter	Value	Unit			
Mean	1.00				
Standard deviation	0.10				
Minimum	0.80				
Maximum	1.20				
Range	0.40				
Skewness	0.00				
Kurtosis	0.00				
Mean square	0.01				
Adjusted R square	0.00				
Standard error of estimate	0.10				
Sum of squares	0.01				
Regression	0.01				
Residual	0.00				
Total	0.01				
Corrected total	0.01				
ANOVA					
Source	Sum of Squares	df	Mean Square	F	Sig.
Regression	0.01	1	0.01	0.01	0.93
Residual	0.00	1	0.00	0.00	1.00
Total	0.01	2			
Corrected Total	0.01	1			

- NOTE:** According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

**WARNING:** *"The claims of a new application may be finally rejected in the first Office action in those situations where (A) the new application is a continuing application of, or a substitute for, an earlier application, and (B) all the claims of the new application (1) are drawn to the same invention claimed in the earlier application, and (2) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 7th ed.*

**NOTE:** Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

☐ Applicant has established small entity status by the filing of a statement in parent application /\_\_\_\_\_ on \_\_\_\_\_.  
☐ A copy of the statement previously filed is included.

**WARNING:** "Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 7th ed. (emphasis added).

☐ A notification of the filing of this  
(check one of the following)

- is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

(Added Pages for Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed  
[4-1.1]—page 5 of 5)



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
NEW PATENT APPLICATION**

**TITLE:** AMINO-TERMINALLY TRUNCATED RANTES AS  
CHEMOKINE ANTAGONISTS

**INVENTORS:** Paul PROOST  
Sofie STRUYF  
Jo VAN DAMME

**ATTORNEY:** Peter F. Corless (Reg. No. 33,860)  
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Fax: (617) 523-6440

008210" 00020560

## AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

### FIELD OF THE INVENTION

The present invention relates to amino-terminally truncated RANTES, lacking  
5 NH<sub>2</sub>-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of  
the naturally-occurring RANTES and having chemokine antagonistic activity, as well as  
cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases,  
in which an antagonistic activity of the chemokine effects is required, and  
pharmaceutical compositions comprising them.

### BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with  
leukocyte chemotactic and activating properties. Depending on the position of the first  
cysteines, the chemokine family can be divided in C-C, C-X-C and C-X<sub>3</sub>-C chemokines  
15 (Baggiolini M. et al., 1994; Baggiolini M. et al., 1997 and Taub D. et al., 1996).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for  
neutrophils, while C-C chemokines, such as monocyte chemotactic protein-3 (MCP-3),  
are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils,  
basophils, NK cells and dendritic cells.

20 The NH<sub>2</sub> terminal domain of chemokines is involved in receptor-binding and  
NH<sub>2</sub>-terminal processing can activate chemokines, reduce their chemokine activity or  
render chemokines completely inactive.

The C-X-C chemokine platelet basic protein becomes a neutrophil chemotactic  
peptide (NAP-2) only after removal of the 24 NH<sub>2</sub>-terminal residues (Walz A. et al.,  
25 1989 and Van Damme J. et al., 1990).

Deletion of up to 8 NH<sub>2</sub>-terminal residues from IL-8 results in an enhanced  
chemotactic activity, but further cleavage of the Glu-Leu-Arg motif, which is located in  
front of the first Cys in all neutrophil chemotactic C-X-C chemokines, causes complete  
inactivation (Clark-Lewis I. et al., 1991).

Similar NH<sub>2</sub>-terminal proteolysis (up to 8 amino acids) of another C-X-C chemokine, granulocyte chemotactic protein-2 (GCP-2), has no effect on the neutrophil chemotactic activity (Proost P. et al, 1993a).

RANTES (is an acronym for "Regulated upon Activation, Normally T Expressed, and presumably Secreted") is a C-C chemokine, whose cDNA clone has been isolated from a cDNA library enriched for T cell-specific sequences (Schall T. J. et al., 1988).

The synthetical C-C chemokines MCP-1, MCP-3 and RANTES missing the 8 to 9 NH<sub>2</sub>-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong J. et al., 1996; and Gong J. et al., 1995).

Extension of RANTES with one methionine results in complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic RANTES (Proudfoot A.E. et al., 1996).

#### DESCRIPTION OF THE INVENTION

The main object of the present invention is amino-terminally truncated RANTES, lacking NH<sub>2</sub>-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity.

A particular object of the present invention is RANTES(3-68), which is RANTES lacking the first 2 amino acids, as shown in Figure 1 and in SEQ ID NO: 2.

The amino-terminally truncated RANTES of the invention can be in a glycosylated or non-glycosylated form.

The term "chemokine antagonist" means 'which acts as antagonist to the mature full-length naturally-occurring chemokines'.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the amino-terminally truncated RANTES of the invention, including nucleotide sequences substantially the same. The cDNA sequence of intact RANTES is disclosed in Schall T. J. et al. (1988) and the cDNA of the truncated RANTES can be easily deduced.

"Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

5           The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early

The DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells, that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to “shuttle” the vector between host cells of different species.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high

copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

The amino-terminally truncated RANTES of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, utilizing automated solid-phase peptide synthesizers followed by chromatographic purification.

The chemokines of the invention may, for example, be synthesized by Fmoc (9-fluorenylmethoxycarbonyl), tBoc (t-butoxycarbonyl) or any other comparable chemical synthesis with or without appropriate side-chain protection groups on the different amino acids. The amino acids with or without appropriate side-chain protection groups are preactivated - e.g. with HBTU/HOBt [2-(1H-Benzotriazole-1yl)-1,1,3,3-tetramethyl-uromium hexafluorophosphate/1-hydroxybenzotriazole) - and coupled to the growing peptide chain. Before the addition of the following residue, the protection group (e.g. Fmoc) is removed from the  $\alpha$ -amino group. After synthesis, all protection groups are removed, the intact full length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokines of the invention.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like (see for example Proost P. et al., 1996). A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, or affinity for heparin, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The amino-terminally truncated RANTES of the invention are useful in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required. Examples of such diseases include: inflammatory diseases, angiogenesis- and hematopoiesis-related diseases, tumors, infectious diseases, including HIV, auto-immune diseases, atherosclerosis, pulmonary diseases and skin disorders. The preferred use is in the field of HIV-infection.

Therefore, in a further aspect, the present invention provides the use of the protein of the invention in the manufacture of a medicament for the treatment of the above-mentioned diseases.

The medicament is preferably presented in the form of a pharmaceutical composition comprising the proteins of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

A further embodiment of the invention is the method of treatment of the above-mentioned diseases comprising administering a pharmacologically active amount of the amino-terminally truncated RANTES of the invention to subjects at risk of developing such diseases or to subjects already showing such pathologies.

It has also been found that CD26/DPP IV is able to generate NH<sub>2</sub>-terminally truncated RANTES in vitro. RANTES is the first cytokine reported whose biological activity can be modified by CD26/DPP IV.

Therefore, another object of the present invention is the use of CD26/DPP IV in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, with particular focus on inflammatory, immune and infectious diseases.

Since this represents the first example of an identified mechanism for endogeneously regulated chemokine modification into an antagonist, similar physiological processing, but mediated by other factors (proteases). The use of such factors (proteases) in the therapy and/or diagnosis of the above diseases is also included in this invention.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

5 DESCRIPTION OF THE FIGURES

Figure 1: it shows the amino acid sequences of RANTES. Signal sequences are reported in *italics*, whereas C -residues are in bold. Arrows indicate the first amino acids of the amino-terminally truncated RANTES of the invention, also called RANTES(3-68).

10 Figure 2: The chemotactic potencies of intact and NH<sub>2</sub>-terminally truncated forms of natural or recombinant RANTES for monocytic THP-1 cells were compared in the Boyden microchamber assay: Natural RANTES(1-68) ( $\Delta$ ), natural, truncated RANTES(3-68) ( $\square$ ), intact recombinant RANTES(1-68) ( $\blacktriangle$ ) and CD26/DPP IV cleaved recombinant RANTES(3-68) ( $\blacksquare$ ). Results represent the mean chemotactic index  $\pm$  SEM of four or more independent experiments.

15 Figure 3: Effect of natural RANTES(3-68) ( $\square$ ), natural RANTES(1-68) ( $\Delta$ ), recombinant RANTES(1-68) ( $\blacktriangle$ ) and recombinant CD26/DPP IV treated RANTES(3-68) ( $\blacksquare$ ) on the  $[Ca^{2+}]_i$  in THP-1 cells. Results represent the mean increase in  $[Ca^{2+}]_i \pm$  SEM of three or more independent experiments.

20 Figure 4: It shows the desensitization of the  $Ca^{2+}$ -mobilizing activity of intact recRANTES(1-68) by RANTES(3-68). THP-1 cells were first stimulated with buffer or different concentrations of recombinant RANTES(1-68) or RANTES(3-68). Results represent the mean  $\pm$  SEM (three or more independent experiments) increase in  $[Ca^{2+}]_i$  in response to 30 ng/ml of intact recombinant RANTES as a second stimulus.

25 Figure 5: Comparison of the chemotactic potency of truncated RANTES(3-68) with intact RANTES(1-68). The eosinophilic granulocyte chemotactic activity of natural (nat) and recombinant (rec) truncated RANTES, intact RANTES and synthetic MCP-3 was determined in the microchamber assay. Results represent the mean chemotactic index (CI)  $\pm$  SEM of two or more independent experiments (each performed in triplicate).

30 Figure 6: Desensitization of calcium mobilization by intact RANTES in CCR transfectants. Calcium mobilization experiments were performed in HOS cells transfected with CD4 and the CC chemokine receptors CCR1 or CCR5. Cells were first stimulated



with different concentrations of intact or truncated RANTES, followed by stimulation with 100 ng/ml of intact RANTES. The percentage inhibition of the  $[Ca^{2+}]_i$  increase induced by the second stimulus is shown. This percentage was calculated by comparing the response of 100 ng/ml of intact RANTES after addition of RANTES(1-68) or RANTES(3-68) with the response after stimulation with buffer (100%). Results represent the mean percentage inhibition  $\pm$  SEM of two or more experiments.

**Figure 7:** Potent inhibitory effect of RANTES (3-68) on infection of mononuclear cells by HIV-1. PHA-activated PBMC were infected with M-tropic HIV-1 Ba-L strain in the presence of various concentrations of RANTES (1-68) or RANTES (3-68) (0 to 1,000 ng/ml added at the time of infection). After ten days virus yields were monitored in the cell supernatant by a p-24 Ag ELISA (one representative experiment out of four is shown).

**Figure 8:** Effects of RANTES(1-68) and RANTES(3-68) on infection by the HIV-1 SF162 strain in PHA-activated PBMC. Virus yields were monitored 10 days after infection by a p24 Ag ELISA on the cell supernatant. Results of a representative experiment out of three are shown. \* Under the detection limit of the p24 Ag ELISA (<5 pg/ml).

**Figure 9:** Expression of CD26 on HOS.CD4.CCR5 cells, U87.CD4.CCR5 cells and freshly-isolated PBMC. The percentage (%) of CD26 positive cells is indicated in each histogram.

**Figure 10:** Effects of RANTES(1-68), RANTES(1-68) plus sCD26 (50 U/L), and RANTES(3-68) on infection of HOS.CD4.CCR5 cells by the HIV-1 BaL strain. Virus yields were monitored in the cell supernatant 8 days after infection by a p24Ag ELISA. Results of a representative experiment out of three is shown.

## EXAMPLES

### EXAMPLE 1: Amino-terminally truncated RANTES

#### *Material and Methods*

#### *Reagents*

Natural human RANTES was produced by human Malavu hepatosarcoma cells, MG-63 osteosarcoma cells or peripheral blood leukocytes (Blood transfusion centers of

Antwerp and Leuven) and purified as previously described (Proost P. et al., 1996 and Proost P. et al., 1993). MCP-2, MCP-3 and GCP-2 were synthesized by Fmoc chemistry (Proost P. et al., 1995 and Wuyts A. et al., 1997), recombinant human RANTES was obtained from Peprotech (Rocky Hill, NJ) and recombinant MCP-1 was a gift from Dr. J.J. Oppenheim (NCI-NIH, Frederick, MD).

Human osteosarcoma (HOS) cells transfected with CD4 and one of the CC chemokine receptors CCR1, CCR3 or CCR5 (Deng H., et al., 1996) were grown in DMEM with glutamax. Puromycin (1 µg/ml) was added to the medium as a selection agent. All growth media (Gibco BRL/Life Technologies, Paisley, UK) were enriched with 10% FCS.

Human CD26/DPP IV was obtained from prostasomes, prostate derived organelles, which occur freely in seminal plasma. The enzyme was purified to homogeneity as described before using ion exchange followed by affinity chromatography onto adenosine deaminase (De Meester I. et al., 1996).

#### *Incubation of chemokines with CD26/DPP IV and detection of proteolytic processing*

A 100 to 1000 molar excess of chemokine was incubated overnight with CD26/DPP IV in 100 mM Tris/HCl pH 7.7. Chemokines were separated from CD26/DPP IV by SDS-PAGE on a Tris/Tricine gel system as previously described (Proost P. et al., 1996).

Proteins were electroblotted on PVDF (polyvinylidene fluoride) membranes (Problott, Perkin Elmer, Foster City, CA) and stained with coomassie brilliant blue R250. After destaining, membranes were rinsed at least 5 times with ultrapure water (Milli Q; Millipore, Bedford, MA).

To obtain sufficient amounts of pure truncated chemokine for biological assays, about 50 µg of recombinant chemokine was treated with CD26/DPP IV and the cleavage product was acidified with 0.1 % trifluoroacetic acid (TFA). Tween 20 (0.01 %) was added to prevent the chemokines from sticking to the tubes.

Chemokines were separated from CD26/DPP IV in an acetonitrile gradient on a C-8 Aquapore RP-300 column (1 x 50 mm) (Perkin Elmer). Fractions containing proteins were analyzed by SDS-PAGE and silver stained as described.

CD26/DPP IV treated chemokines, purified by RP-HPLC or excised from PVDF blots, were NH<sub>2</sub>-terminally sequenced by Edman degradation on a pulsed liquid phase 477A/120A protein sequencer (Perkin Elmer) using *N*-methylpiperidine as a coupling base.

#### *Detection of chemotactic activity*

Chemokines were tested for their chemotactic potency on freshly isolated peripheral blood neutrophilic granulocytes (10<sup>6</sup> cells/ml) or cultured monocytic THP-1 cells (0.5 x 10<sup>6</sup> cells/ml) in the Boyden microchamber (Proost P. et al., 1996 and Proost P. et al., 1993).

After 45 min (granulocytes) or 2 h (THP-1 cells) incubation at 37 °C, the cells were fixed and stained. The cells that migrated through the 5 µm pore size polycarbonate membranes were counted microscopically in ten oil immersion fields.

The chemotactic index (C.I.) of a sample (triplicates in each chamber) was calculated as the number of cells that migrated to the test sample divided by the number of cells that migrated to control medium. In desensitization experiments, cells were incubated with biologically inactive chemokine-variants for 10 min at 37 °C before transfer to the chamber.

The percentage inhibition of the C.I. obtained by desensitization with HBSS-treated control cells was calculated for the evaluation of chemotaxis desensitization.

#### *Detection of intracellular Ca<sup>2+</sup> concentrations*

Intracellular Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) were measured as previously described (Wuyts A., et al., 1997). Briefly, purified cells were incubated with the fluorescent indicator fura-2 (2.5 µM fura-2/AM, Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01 % Pluronic F-127 (Sigma, St. Louis, MO).

After 30 min, cells were washed twice, resuspended in HBSS with 1 mM Ca<sup>2+</sup> and incubated for 10 min at 37 °C before fura-2 fluorescence was measured in an LS50B

luminescence spectrophotometer (Perkin Elmer). Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The  $[Ca^{2+}]_i$  was calculated from the Grynkiewicz equation (Grynkiewicz G. et al., 1985).

In order to determine  $R_{max}$ , the cells were lysed with 50  $\mu$ M digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and  $R_{min}$  was obtained by addition of 10 mM EGTA to the lysed cells. The  $K_d$  used for calibration was 224 nM.

For desensitization experiments, cells were first stimulated with buffer or chemokine at different concentrations. As a second stimulus, chemokines were added at a concentration inducing a significant increase in the  $[Ca^{2+}]_i$  after prestimulation with buffer. The percentage inhibition of the  $[Ca^{2+}]_i$ -increase in response to the second stimulus by prestimulation of the cells was calculated.

#### *Inhibition of HIV-1 infection*

The HIV-1 M-tropic strains BaL and SF162 were obtained through the MRC AIDS reagent project (Herts, UK). Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation (5,23) and stimulated with PHA at 1  $\mu$ g/ml (Sigma, Bornem, Belgium) for 3 days at 37°C. The activated cells (PHA-stimulated blasts) were washed three times with PBS, and infected with a virus as described previously (Schols D. et al., 1997). HIV-1 infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/ml of IL-2 and varying concentrations of RANTES (1-68) or RANTES (3-68). Cell supernatant was collected at day 10 and HIV-1 core antigen in the supernatant was analysed by a p-24 Ag ELISA kit (DuPont/NEN Life Science Products, Brussels, Belgium).

#### *Results*

##### *Identification and biological characterization of natural, NH<sub>2</sub>-terminally truncated RANTES.*

A different NH<sub>2</sub>-terminally truncated form of human GCP-2 has been previously isolated (Proost P. et al., 1993). The least truncated GCP-2-form was cleaved beyond Pro at the penultimate position [GCP-2(3-75)]. Using a similar standard purification

procedure, the C-C chemokine RANTES was purified from peripheral blood leukocytes or sarcoma cells (Proost P. et al., 1996).

In particular, conditioned media from MG-63 or Malavu sarcoma cells induced with a cytokine mixture were fractionated to isolate natural chemokine variants. The chemokines were purified by subsequent antibody or heparin affinity chromatography, cation-exchange chromatography (mono S FPLC) and RP-HPLC, and immunoreactive forms were detected by specific chemokine ELISAs. On the cation-exchange column, IL-8 was found to elute in close proximity of RANTES (between 0.7 and 0.75 M NaCl). Nevertheless, both chemokines were separated from each other by RP-HPLC (RANTES and IL-8 eluting at 27.5% and 30% acetonitrile, respectively). Amino acid sequence analysis of the pure proteins confirmed that IL-8 occurred in different NH<sub>2</sub>-terminally truncated forms, which were previously isolated on the basis of their chemotactic activity (Van Damme J. et al., 1989). However, for RANTES only one single form was isolated, which was missing two NH<sub>2</sub>-terminal residues compared to intact RANTES. In view of its predominant appearance, this RANTES(3-68) was analyzed in more detail to verify its chemotactic activity for monocytes and eosinophils. In particular, RANTES(3-68) was tested for chemotactic and/or intracellular Ca<sup>2+</sup>-releasing activity and their biological potency was compared with that of the respective intact chemokines.

NH<sub>2</sub>-terminal deletion of two residues from RANTES resulted in considerably decreased monocyte chemotactic and Ca<sup>2+</sup>-releasing activities. Compared to intact natural RANTES (minimal effective dose of 3-10 ng/ml), natural RANTES(3-68) was totally inactive when tested at concentrations as high as 300 ng/ml in the Boyden microchamber (Figure 2). In addition, 10 times higher concentrations of natural RANTES(3-68), compared to RANTES(1-68), were necessary to obtain a similar Ca<sup>2+</sup>-response (Figure 3).

#### *CD26/DPP IV removes the NH<sub>2</sub>-terminal dipeptides of chemokines*

In order to investigate whether the aminopeptidase CD26/DPP IV could be responsible for the NH<sub>2</sub>-terminal truncation of RANTES, the intact chemokine was incubated overnight with CD26/DPP IV, blotted to PVDF membranes, stained with Coomassie blue and subjected to automatic Edman degradation. CD26/DPP IV

treatment of RANTES resulted in the removal of the NH<sub>2</sub>-terminal dipeptides. Parallel incubation of chemokine with buffer without CD26/DPP IV had no effect.

Since other chemokines contained the consensus sequence for CD26/DPP IV cleavage and since the NH<sub>2</sub>-terminus of MCPs was shown to be crucial for biological activity (Gong J. et al., 1996 and Gong J. et al., 1995), MCP-1, MCP-2 and MCP-3 were also incubated with CD26/DPP IV.

After treatment, MCPs were blotted on PVDF membranes and Coomassie blue stained to confirm that a sufficient amount of protein was recovered for Edman degradation.

However, no NH<sub>2</sub>-terminal sequence could be detected, indicating that CD26/DPP IV does not alter the NH<sub>2</sub>-terminus of MCPs which is blocked for Edman degradation by a pyroglutamic acid.

#### *Comparison of the biological activity of intact and CD26/DPP IV-treated RANTES.*

Similar to natural RANTES(3-68), C-8 RP-HPLC purified, CD26/DPP IV-treated recombinant RANTES was inactive in Boyden microchamber chemotaxis experiments when used at concentrations up to 1 µg/ml, while a significant monocyte chemotactic response was detected with intact recombinant RANTES from 30 to 100 ng/ml onwards (Figure 2).

When the truncation effect was tested in the Ca<sup>2+</sup>-mobilization assay, RANTES(3-68) induced a low but significant increase at 100 ng/ml. Intact RANTES, however, was already active at 10 ng/ml (Figure 3). In conclusion, although only two NH<sub>2</sub>-terminal residues were removed, the monocyte chemotactic and Ca<sup>2+</sup>-mobilizing potency of RANTES decreased 10 to 100-fold.

#### *RANTES(3-68) is a natural chemotaxis antagonist for intact RANTES*

In view of the inactivity of RANTES(3-68) in monocyte chemotaxis experiments, we tested whether this truncated RANTES might act as an antagonist. RANTES(3-68), at 1 µg/ml, almost completely (82 %) desensitized for the chemotactic effect of 100 ng/ml of intact RANTES (Table I).

When a 3-fold excess of RANTES(3-68) was added to the upper well, chemotaxis of THP-1 cells towards intact RANTES was inhibited by about 50-70 %. RANTES(3-68) at 300 ng/ml could still inhibit about 30 % of the chemotactic response towards an equal concentration of intact RANTES.

- 5        In  $\text{Ca}^{2+}$ -mobilization experiments with THP-1 cells (Figure 4), 30 ng/ml of intact RANTES could desensitize for the effect of 30 ng/ml of intact RANTES for  $39 \pm 5$  %. About ten-fold higher concentrations of RANTES(3-68) were necessary to obtain the same amount of desensitization. However, at 300 ng/ml, RANTES(3-68) by itself gave a significant  $\text{Ca}^{2+}$ -response. This  $\text{Ca}^{2+}$ -response was comparable to the response obtained
- 10      with 30 ng/ml of intact RANTES.

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TABLE I

*RANTES(3-68) desensitizes monocyte chemotaxis induced by RANTES(1-68)<sup>1</sup>*

Chemokine(ng/ml)		Chemotactic response (CI)					
Lower well	Upper well						% inhibition
RANTES (1-68)	RANTES (3-68)	A	B	C	D	mean $\pm$ SEM	mean $\pm$ SEM
300	1000	12.5	7.5	27.5	50.5	25 $\pm$ 10	67 $\pm$ 8
	300	22.0	20.5	72.5	79.5	49 $\pm$ 16	31 $\pm$ 13
	0	41.0	46.0	71.5	97.0	64 $\pm$ 3	0
100	1000	4.0	3.0	13.5	11.0	8 $\pm$ 3	82 $\pm$ 4
	300	7.5	7.0	29.0	33.0	19 $\pm$ 7	53 $\pm$ 11
	0	24.0	21.5	50.0	44.5	35 $\pm$ 7	0

<sup>1</sup> Results represent the chemotactic index (C.I.) of four (A to D) independent experiments (including mean  $\pm$  SEM) and the percentage (%) inhibition (mean  $\pm$  SEM of the % inhibition of the four experiments) of the chemotactic response towards RANTES(1-68) after preincubation of the THP-1 cells with inactive RANTES(3-68) or buffer.

*Impaired chemotactic activity of RANTES(3-68) for human monocytes and eosinophils*

In Table II the chemotactic potency of natural RANTES(3-68) is compared with that of the monocyte chemotactic protein MCP-3 and intact RANTES(1-68). It can be seen that MCP-3 and RANTES(1-68) are still chemotactic for freshly isolated peripheral blood monocytes at 3 ng/ml and 30 ng/ml, respectively, whereas natural RANTES(3-68) remained inactive at 100 ng/ml.

The reduced chemotactic potency of this natural variant, was confirmed with recombinant RANTES(3-68). Although weakly chemotactic for monocytes (at 1  $\mu$ g/ml), purified recombinant RANTES(3-68) showed a specific activity which is 10-fold lower than that of intact recombinant RANTES.

Finally, the chemotactic potency of RANTES(3-68) was verified on human eosinophils, which were still responsive to 100 ng/ml of intact RANTES and 30 ng/ml of



MCP-3 (Figure 5). Similar to monocytes, eosinophil migration was only stimulated by RANTES(3-68) at 1 µg/ml.

TABLE II

5 *Comparison of the monocyte chemotactic activity of RANTES(3-68) with RANTES(1-68) and MCP-3*

monocyte chemotactic activity <sup>a)</sup>				
conc. (ng/ml)	MCP-3	natRANTES (3-68)	recRANTES (1-68)	recRANTES (3-68)
1000	— <sup>b)</sup>	—	3.6 ± 0.8 (6)	3.3 (1)
300	6.0 ± 1.2 (6)	—	—	—
100	—	1.1 ± 0.1 (3)	3.3 ± 0.4 (6)	1.0 (1)
30	6.9 ± 1.0 (6)	1.7 ± 0.2 (3)	—	—
10	—	1.9 ± 0.6 (3)	1.9 ± 0.4 (6)	< 1.0 (1)
3	4.1 ± 0.4 (6)	—	—	—

<sup>a)</sup> mean chemotactic index (CI) ± SEM (n) on freshly isolated peripheral blood monocytes.

<sup>b)</sup> not determined

*RANTES(3-68) signals and desensitizes for RANTES(1-68) through CCR5, but not through CCR1 and CCR3*

15 To explain the reduced chemotactic activity of RANTES(3-68), the capacity of this chemokine variant to bind and signal through the known receptors used by RANTES was verified.

HOS cells transfected with the chemokine receptors CCR1, CCR3 or CCR5 were used in a signaling assay measuring increases in the intracellular calcium concentration. At concentrations up to 300 ng/ml, RANTES(3-68) did not increase the [Ca<sup>2+</sup>]<sub>i</sub> in HOS cells transfected with CCR1 (Table III) or CCR3 (data not shown), whereas 30 ng/ml and 100 ng/ml of intact RANTES was sufficient to induce an increase in [Ca<sup>2+</sup>]<sub>i</sub> in CCR1 and CCR3 transfectants, respectively.

20 However, both intact and truncated RANTES were able to induce a significant rise in [Ca<sup>2+</sup>]<sub>i</sub> in CCR5 transfectants at 30 ng/ml. Furthermore, by pre-incubation of

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CCR5-transfected cells with a 3 to 10-fold excess of either RANTES(3-68) or intact RANTES, an equal inhibition (about 75%) of the  $[Ca^{2+}]_i$  rise by a subsequent challenge with intact RANTES (100 ng/ml) was obtained (Figure 6).

In contrast, 300 ng/ml of RANTES(3-68) only marginally desensitized the calcium response of CCR1 and CCR3-transfected cells to 100 ng/ml of intact RANTES, whereas a 3-fold excess of intact RANTES as first stimulus almost completely inhibited the  $[Ca^{2+}]_i$  rise in these cells by subsequent RANTES(1-68). It must be concluded that removal of two  $NH_2$ -terminal residues from RANTES has a significant impact on signal transduction in that the chemokine receptors CCR1 and CCR3 are no longer functionally recognized. Therefore, the impaired chemotactic potency of RANTES(3-68) can be explained by its inability to function through CCR1 and CCR3. In contrast, RANTES(3-68) fully retained the CCR5 signalling characteristic of intact RANTES. RANTES(3-68) can be anti-inflammatory by competing with intact RANTES, but may still function as an HIV-inhibitor by retaining its capacity to bind CCR5.

TABLE III

*Calcium mobilization by RANTES forms in CCR1 and CCR5 transfectants*

chemokine	conc. (ng/ml)	increase in $[Ca^{2+}]_i$ (nM) <sup>a)</sup>	
		CCR1	CCR5
RANTES(1-68)	300	133 ± 5 (3)	96 ± 1 (2)
	100	100 ± 28 (3)	60 ± 4 (2)
	30	25 ± 8 (3)	24 ± 2 (2)
	10	<15 (2)	<15 (1)
RANTES(3-68)	300	19 ± 9 (3)	119 ± 5 (2)
	100	<15 ± 0 (3)	76 ± 4 (2)
	30	<15 (2)	56 ± 13 (2)
	10		<15 (1)

<sup>a)</sup> the mean increase in  $[Ca^{2+}]_i$  in nM ± SEM of two or more independent experiments is shown.

*Inhibition of CC chemokine-induced chemotaxis by RANTES(3-68) in human monocytic cells*

To verify whether inhibition of CC chemokine signaling by RANTES(3-68) also occurred in monocytic cells, inhibition experiments were conducted in THP-1 cells. It was evidenced that RANTES(3-68) showed a 10-fold reduction in potency to increase the  $[Ca^{2+}]_i$  in monocytic cells compared to intact RANTES (data not shown). In addition, the chemotactic effect of intact RANTES (30 ng/ml) on monocytic cells was inhibited (71%) by incubating the test cells with 300 ng/ml RANTES(3-68) as shown in Table IV.

Furthermore, RANTES(3-68) reduced the chemotactic response to other CC chemokines, including monocyte chemotactic protein-3 (MCP-3) (67%), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) (61%) and MIP-1 $\beta$  (80%).

This illustrates that RANTES(3-68) functions as a broad spectrum inhibitor of monocytic cell migration induced by other CC chemokines.

TABLE IV

*Inhibition of monocytic cell chemotaxis towards CC chemokines by RANTES(3-68)*

inhibition of THP-1 cell chemotaxis				
chemokine <sup>a)</sup>	conc. (ng/ml)	buffer <sup>b,c)</sup>	RANTES(3-68) <sup>b,c)</sup>	% inhibition <sup>d)</sup>
RANTES	30	19.0 $\pm$ 6.6	3.7 $\pm$ 0.6	71 $\pm$ 16
MCP-3	30	48.5 $\pm$ 9.3	24.9 $\pm$ 2.0	45 $\pm$ 10
MCP-3	3	7.6 $\pm$ 2.5	3.1 $\pm$ 0.8	67 $\pm$ 13
MIP-1 $\alpha$	30	6.2 $\pm$ 2.4	3.0 $\pm$ 1.1	61 $\pm$ 22
MIP-1 $\beta$	300	4.3 $\pm$ 1.0	1.9 $\pm$ 0.6	80 $\pm$ 12
control		1.5 $\pm$ 0.5	1.0 $\pm$ 0.5	

a) RANTES, MCP-3, MIP-1 $\alpha$ , MIP-1 $\beta$  and buffer were added as chemoattractants to the lower wells of the microchamber.

b) the upper wells of the microchamber were filled with THP-1 cells preincubated (10 min, 37°C) with 300 ng/ml RANTES(3-68) or with buffer.

c) mean CI  $\pm$  SEM of four independent experiments.

d) inhibition of migration induced by intact chemokines in the presence of RANTES(3-68) at 300 ng/ml.

*CD26-specific truncation of RANTES is necessary for its antiviral activity.*

The effects of the different forms of RANTES were first evaluated against two different M-tropic HIV-1 strains (BaL and SF162) in human PBMC derived from healthy blood donors. The IC<sub>50</sub> of the intact RANTES(1-68) against the BaL strain was 3.4 nM and for RANTES(3-68) the IC<sub>50</sub> was 0.39 nM. The IC<sub>90</sub> value for RANTES(1-68) was 71 nM, which was about 10-fold the IC<sub>90</sub> for RANTES(3-68). Against the SF162 strain when evaluated in PBMC, RANTES(1-68) (IC<sub>50</sub>: 23 nM; IC<sub>90</sub> : 95 nM) was more than 10-fold less active than RANTES(3-68) (IC<sub>50</sub>: 2 nM; IC<sub>90</sub> : 8.2 nM) (Table V). The concentration-dependent effects of both chemokines at concentrations ranging from 133 down to 0.2 nM against HIV-1 SF162 replication in PBMC are shown in Figure 8. A concentration of 5.2 nM RANTES(3-68) was clearly effective in reducing virus replication, whereas RANTES(1-68) was inactive at this concentration. No difference in antiviral activity was noticed between intact RANTES obtained from PeproTech or R&D Systems.

The striking difference in antiviral activity between the two forms of RANTES became even more apparent when tested in the human CCR5 transfected cells. In U78.CD4.CCR5 cells, the IC<sub>50</sub> for RANTES(1-68) and RANTES(3-68) against the BaL strain was 21 nM and 0.65 nM, respectively. The IC<sub>90</sub> for RANTES(1-68) was more than 133 nM, whereas the IC<sub>90</sub> for RANTES(3-68) was 63 nM. Also in Table V, it is shown that RANTES(1-68) was virtually inactive in the HOS.CD4.CCR5 cells (IC<sub>50</sub> > 133 nM), whereas RANTES(3-68) is a potent inhibitor of HIV-1 BaL replication in these cells (IC<sub>50</sub>: 5.5 nM). However, no IC<sub>90</sub> values were reached for both forms of RANTES in these cells.

*The antiviral activity of RANTES is dependent on the presence of membrane bound or soluble CD26 (sCD26).*

The CD26 expression on the two different CCR5-transfected cell lines and on freshly isolated PBMC was evaluated. HOS transfectants were negative for CD26 expression as determined by flow cytometric analysis, whereas U87 transfectants stained weakly but significantly positive with the anti-CD26 mAb (Figure 9). In addition, a

subpopulation of freshly isolated PMBC was found to be strongly positive for CD26 expression (Figure 9).

The concentration-dependent effect of RANTES(1-68) and RANTES(3-68) on viral p24 Ag production by the BaL strain in HOS.CD4.CCR5 transfected cells in presence of sCD26 is shown in Figure 10. Addition of sCD26, together with RANTES(1-68) at the start of the HIV infection, significantly enhanced the antiviral activity of the intact RANTES in HOS.CD4.CCR5 cells. When sCD26 at 50 U/l was added together with RANTES, an  $IC_{50}$  of 13 nM of RANTES was obtained. The addition of sCD26 alone had no effect on virus replication. The addition of sCD26 to RANTES(3-68) did also not change the antiviral activity of RANTES(3-68) (data not shown). Thus, the presence of CD26 is essential for intact RANTES to become antivirally active.

*Amino terminal truncation of natural MIP-1 $\alpha$  does not affect its anti-HIV-1 chemotactic and  $Ca^{2+}$  mobilising activity.*

Since the majority of natural MIP-1 $\alpha$  is  $NH_2$  terminally truncated (four amino acids), we investigated whether this truncated MIP-1 $\alpha$ (5-70) had an altered HIV-1 inhibitory capacity. In contrast with the results obtained for RANTES, no significant differences were detected for the  $IC_{50}$  values of intact MIP-1 $\alpha$  and MIP-1 $\alpha$ (5-70) in PMBC or CCR5-transfected cells (Table V, Figure 8). In addition, intact MIP-1 $\alpha$  and truncated MIP-1 $\alpha$ (5-70) were compared in chemotaxis and intracellular  $Ca^{2+}$ -mobilization assays on THP-1 monocytic cells. Table VI demonstrates that the minimal effective dose of MIP-1 $\alpha$ (5-70) inducing a rise in the  $[Ca^{2+}]_i$  was only slightly lower than intact MIP-1 $\alpha$ . Furthermore, although maximal migration obtained with 0.13 nM in the chemotaxis assay was higher for intact MIP-1 $\alpha$ , the minimal affective concentrations of both MIP-1 $\alpha$  isoforms were rather similar. Taken together, it must be concluded that  $NH_2$  terminal processing of MIP-1 $\alpha$  in contrast to RANTES, only minimally weakens its inflammatory and anti-HIV-1 activity.

**TABLE V.** Anti-HIV-1 activity of RANTES and MIP-1 $\alpha$  in PHA-stimulated PMBC, U87.CD4.CCR5 cells.

	RANTES(1-68)		RANTES(3-68)		MIP-1 $\alpha$ (1-70)		MIP-1 $\alpha$ (5-70)	
	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>	IC <sub>50</sub>	IC <sub>90</sub>
<b>PMBC</b>								
BaL	3.4	71	039	6.9	1.9	62	1.6	13
SF162	23	95	2.0	8.2	3.1	30	3.6	32
<b><u>U87.CD4.CCR5</u></b>								
BaL	21	>130	0.65	63	ND	ND	ND	ND
<b><u>HOS.CD4.CCR5</u></b>								
BaL	>130	>130	5.5	>130	32	>130	21	>130

5

Virus yield was monitored in the cell-free supernatant 8-12 days after infection by viral p24 Ag ELISA. The mean IC<sub>50</sub>s and IC<sub>90</sub>s (in nM) are shown. The data represent the means of two to four independent experiments. The value marked by ">130" indicates that 50% or 90% inhibition is not achieved at 130 nM. ND, not done.

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**TABLE VI** Lack of difference in biological potency between intact and truncated MIP-1 $\alpha$ .

Chemokine	Chemotaxis*		Increase in $[Ca^{2+}]_i^{\dagger}$	
	nM	C.I	nM	nM $[Ca^{2+}]_i$
MIP-1 $\alpha$ (1-70)	1.3	$8.5 \pm 3.3$	3.9	240/195
	0.13	$22.2 \pm 4.4$	0.39	120/130
	0.013	$5.7 \pm 1.6$	0.34	30/14
	0.0013	$4.0 \pm 3.1$		
MIP-1 $\alpha$ (5-70)	1.3	$14.2 \pm 0.4$	4.0	196/178
	0.13	$11.4 \pm 2.9$	0.4	71/33
	0.013	$3.8 \pm 1.4$	0.04	10/<10
	0.0013	$2.1 \pm 0.6$		

\* Migration of monocyte THP-1 cells through 5.0  $\mu$ m pores in the microchamber. Results are mean  $\pm$  of three independent experiments.

$\dagger$  Detection of the  $[Ca^{2+}]_i$  increase in THP-1 cells. Results of two independent experiments are shown.

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CLAIMS

1. Amino-terminally truncated RANTES, lacking NH<sub>2</sub>-terminal amino acids  
corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring  
5 RANTES and having chemokine antagonistic activity.
2. Amino-terminally truncated RANTES according to claim 1, lacking NH<sub>2</sub>-terminal  
amino acids corresponding to amino acid residues 1-2 of the naturally-occurring  
RANTES and having a chemokine antagonistic activity  
10
3. Amino-terminally truncated RANTES according to claim 1, having the amino acid  
sequence of SEQ ID NO: 2.
4. Amino-terminally truncated RANTES, according to one or more of the preceding  
15 claims, in a glycosylated form.
5. DNA molecules comprising the DNA sequences coding for the amino-terminally  
truncated RANTES of the invention according to one or more of the preceding  
claims, including nucleotide sequences substantially the same.  
20
6. An expression vector which comprises the DNA molecule of any claim 5.
7. A host cell comprising the expression vector of claim 6.
- 25 8. A recombinant process for preparing any of the proteins from claim 1 to 4, comprising  
culturing in an appropriate culture medium the cells of claim 7.
9. A protein according to any of the claims from 1 to 4 for use as medicament.

11. Use according to claim 10, in the manufacture of a medicament for the treatment of inflammatory diseases, HIV-infection, angiogenesis- and hematopoiesis-related diseases, and tumors.

13. Use of CD26/DPP IV in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required.

15

14. Use according to claim 13, for the treatment of inflammatory, immune and infectious diseases.

## ABSTRACT

[illegible]

RANTES

-23

1 ↓

MKVSAARLAV ILIATALCAP ASAPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP  
AVVFEVTRKNR QVCANPEKKW VREYINSLEM S

68

Figure 1

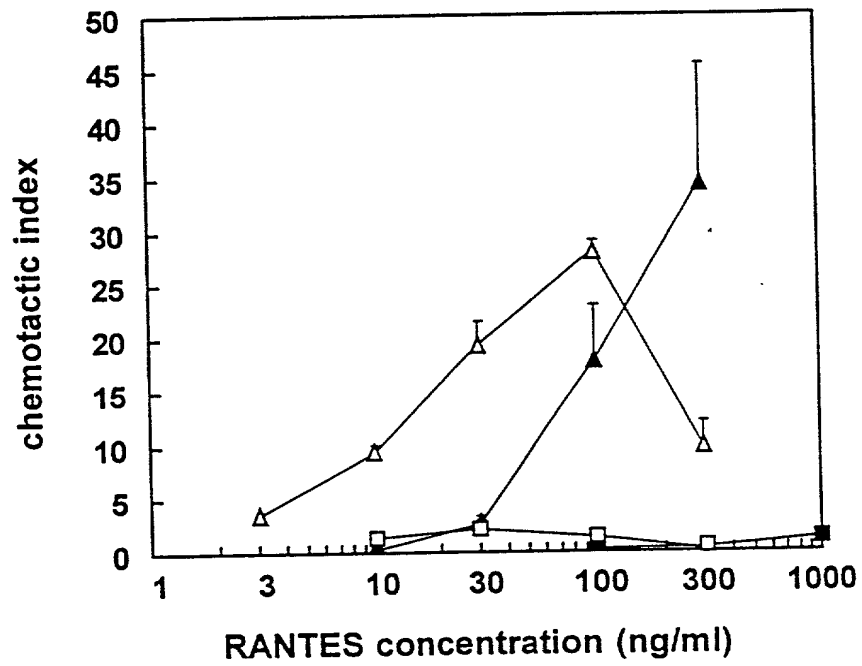


Figure 2

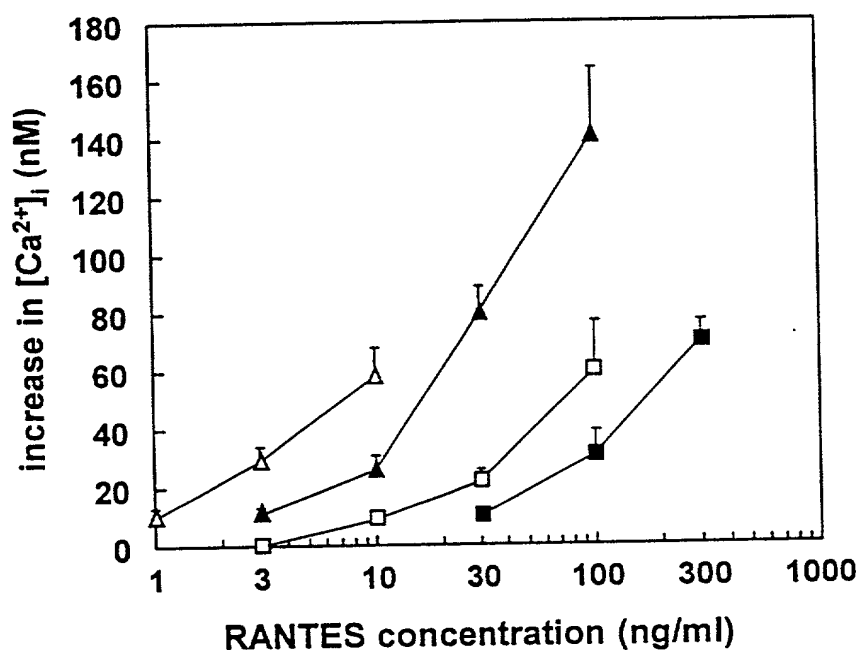


Figure 3

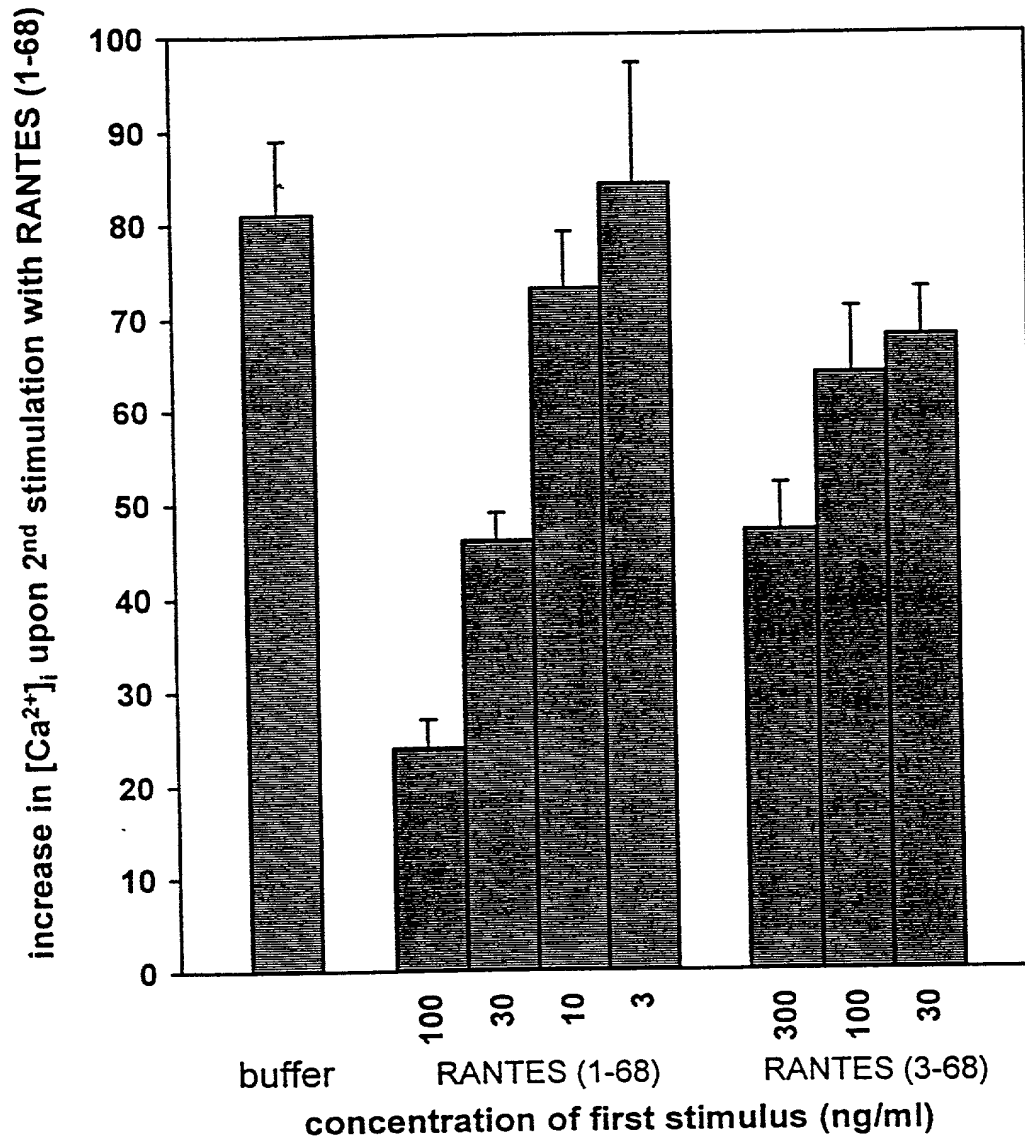


Figure 4

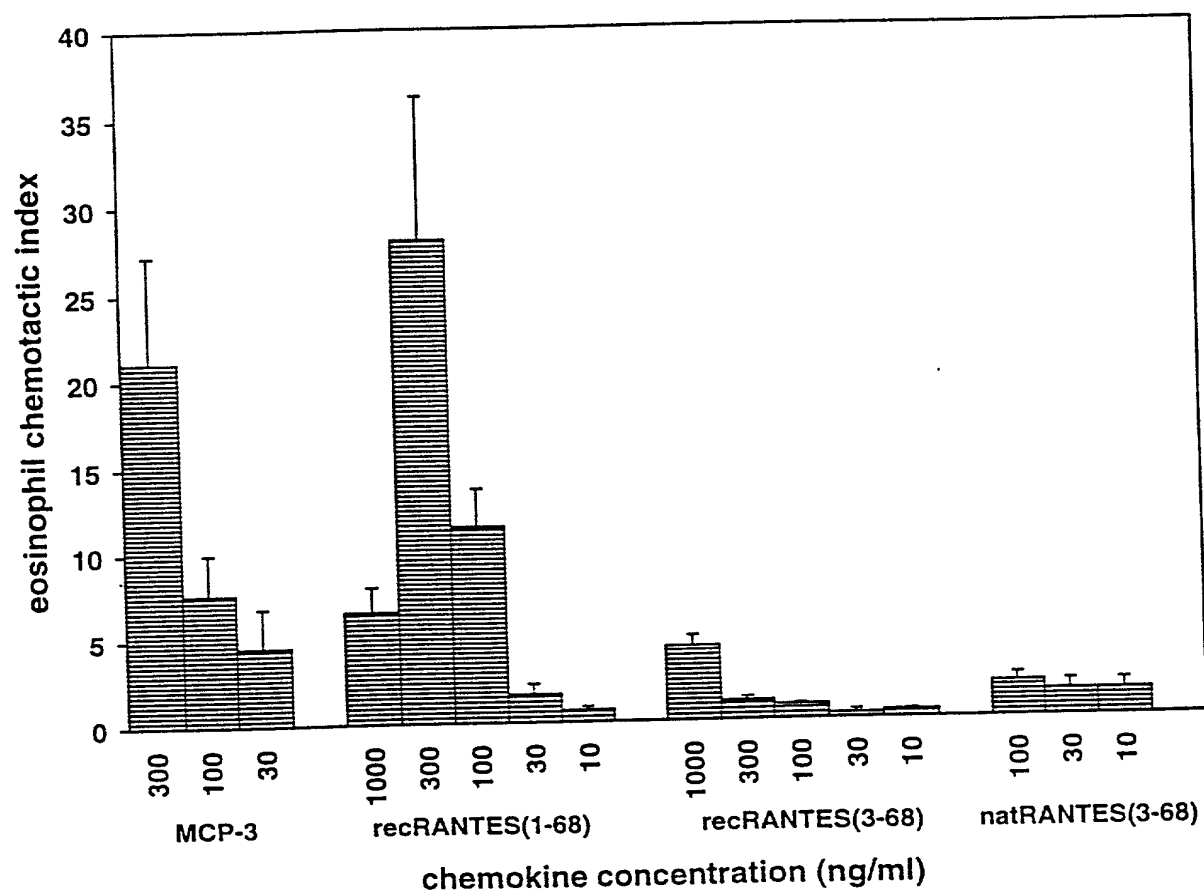


Figure 5



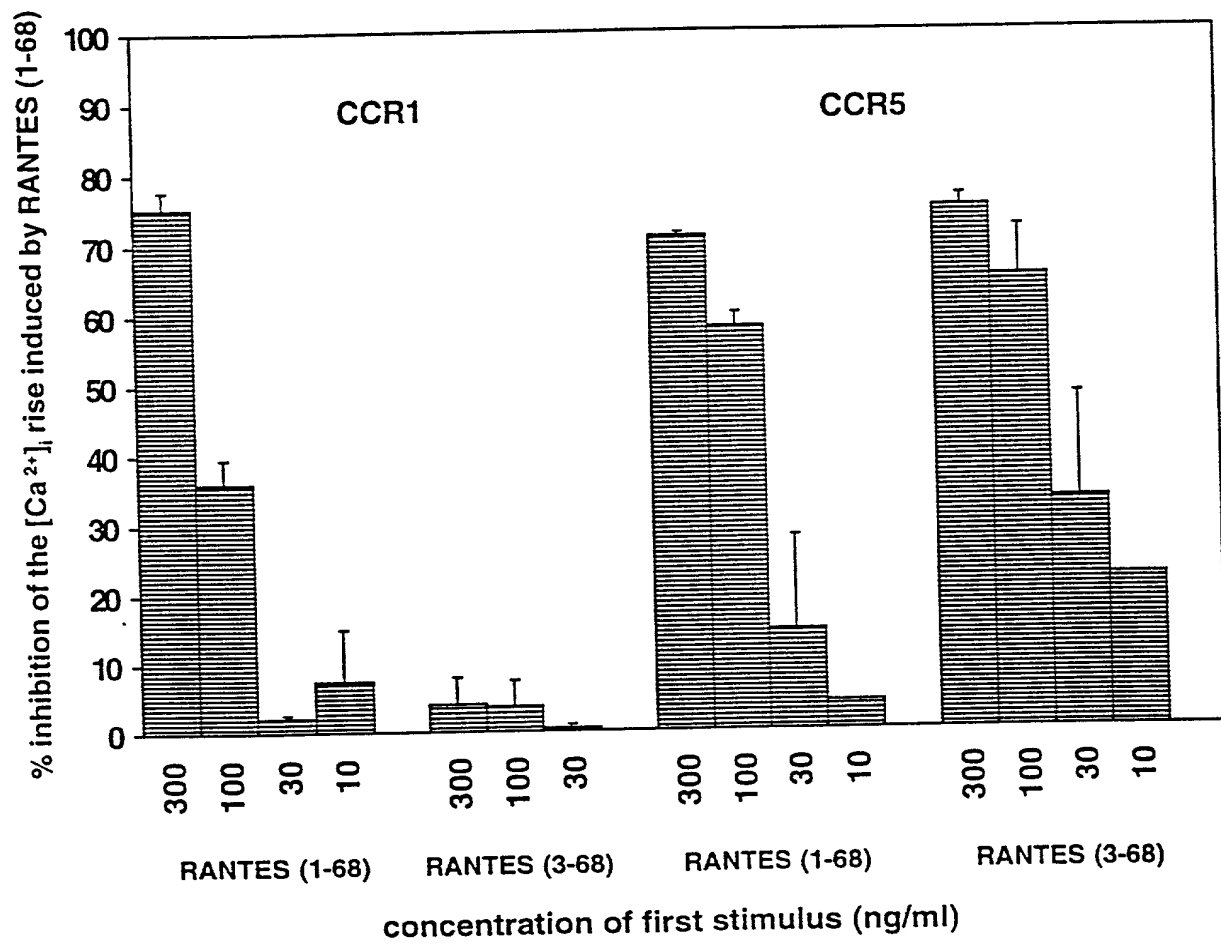


Figure 6

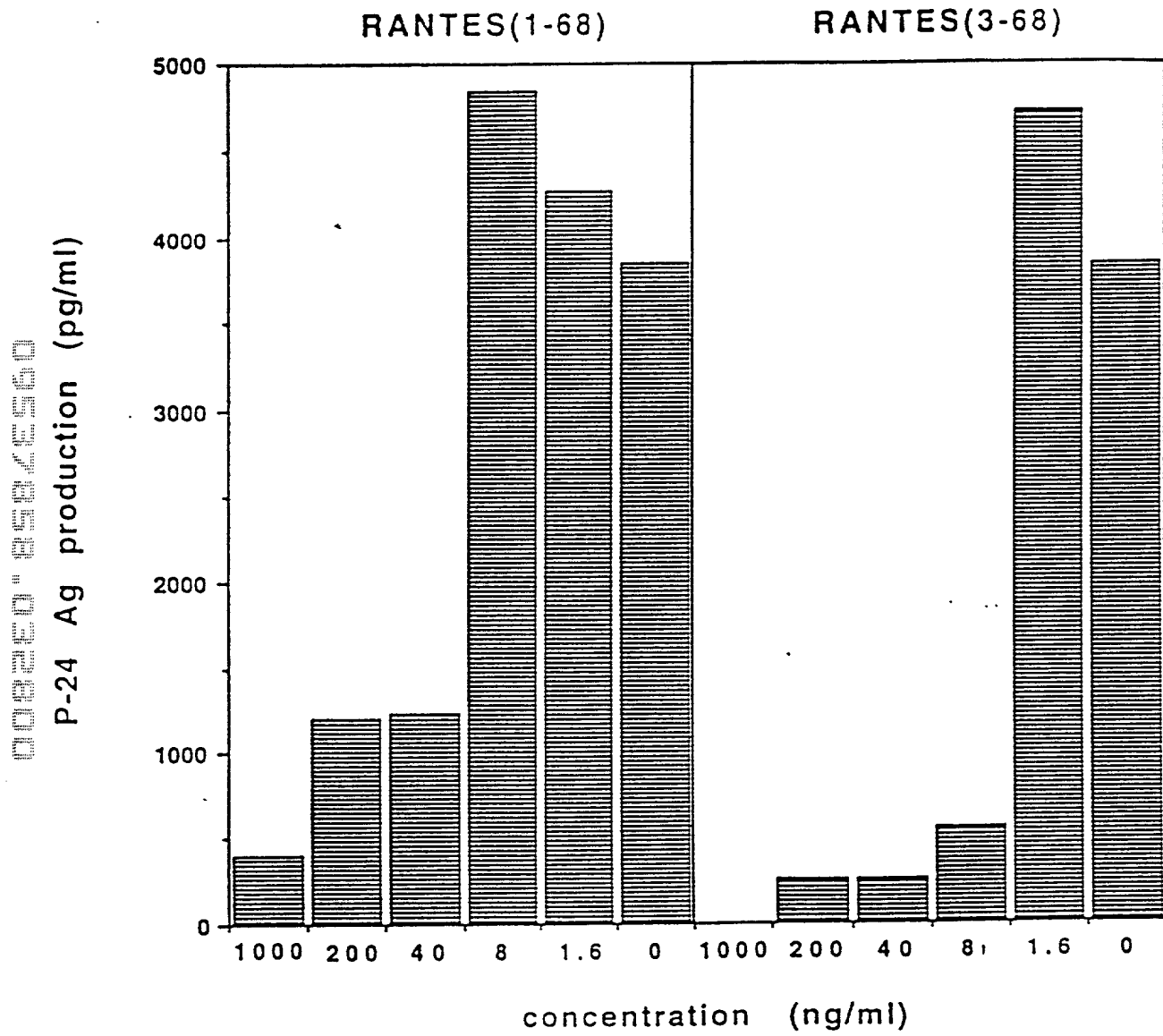


Figure 7

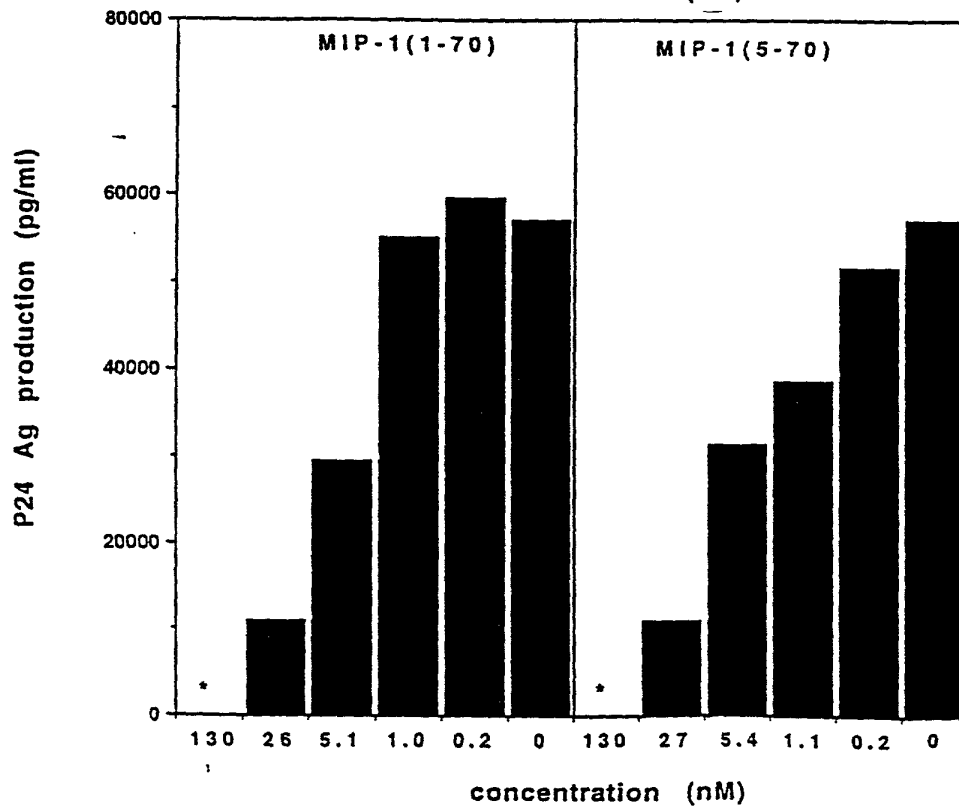
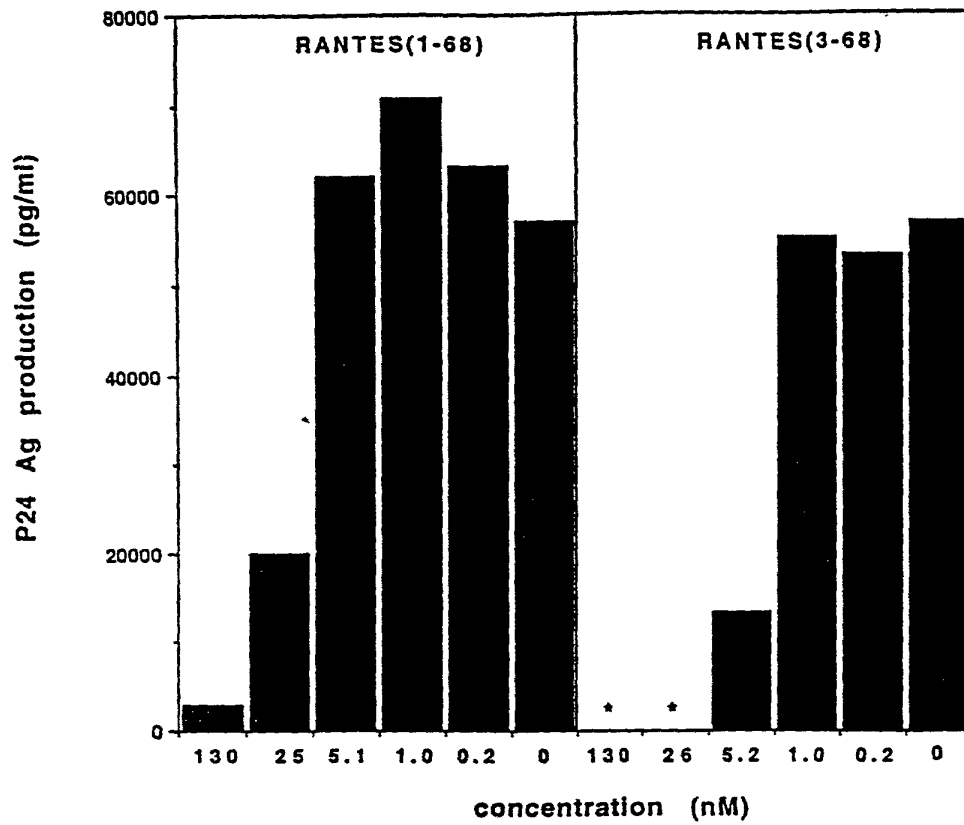


Figure 8

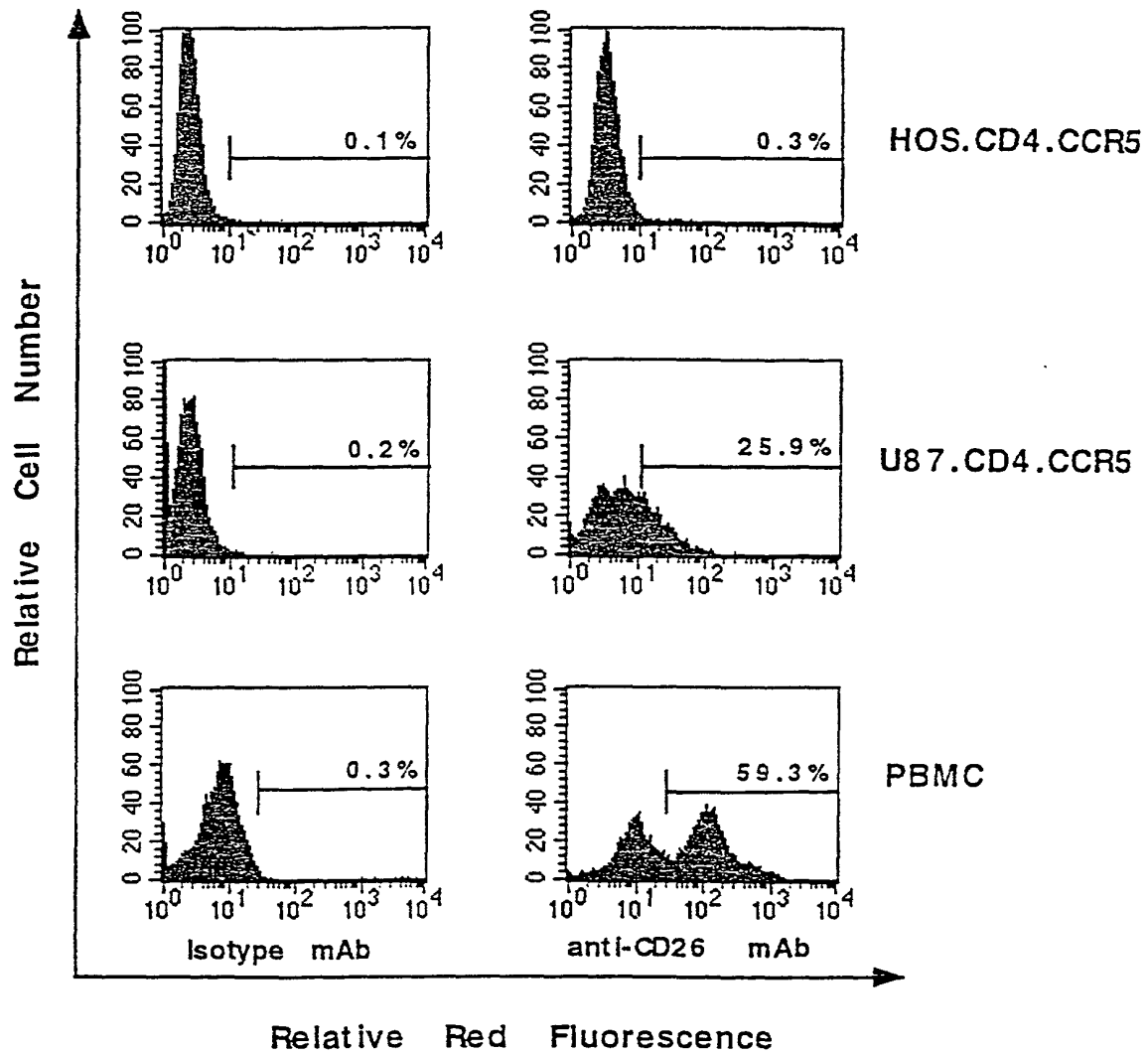


Figure 9

P24 Ag production (pg/ml)

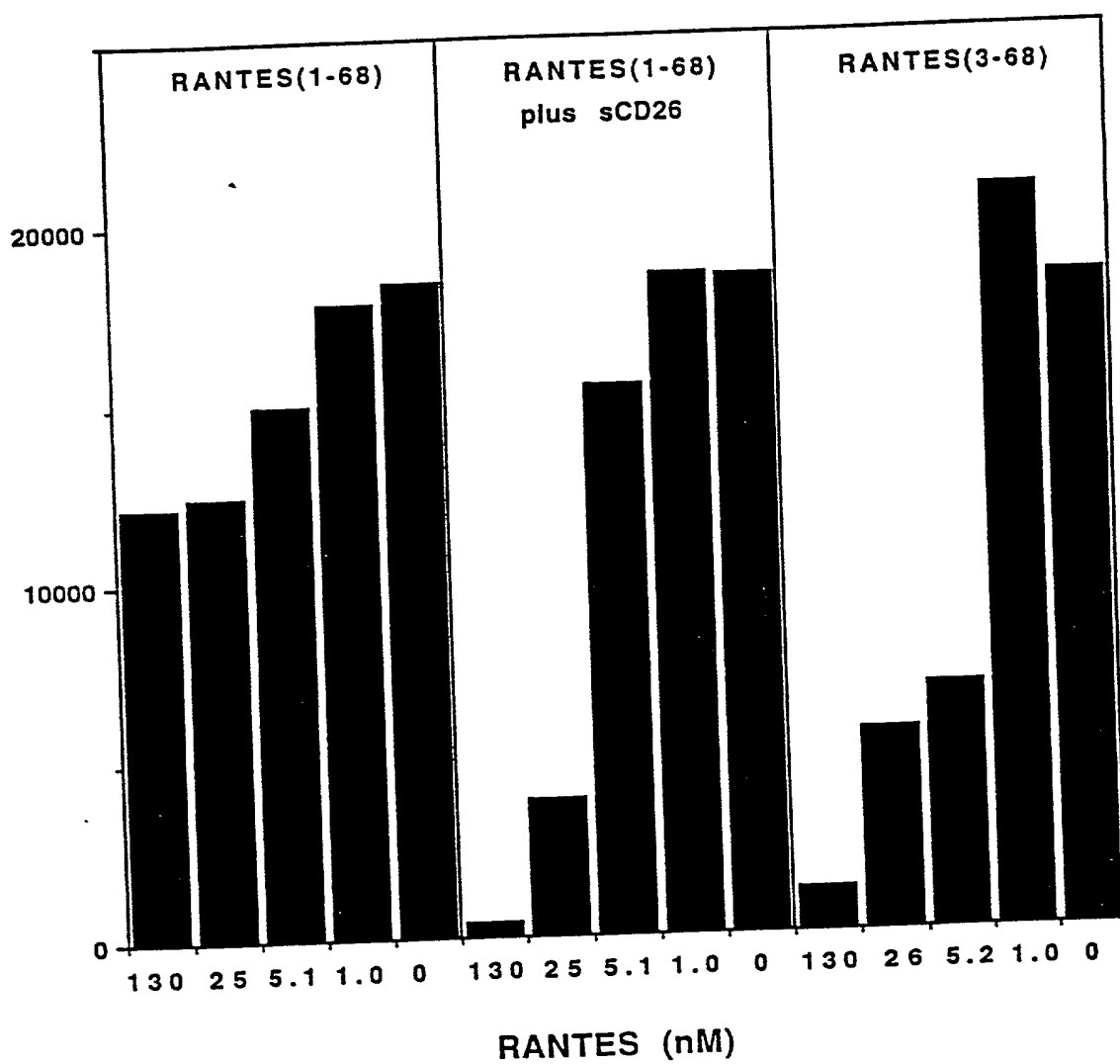


Figure 10

-1-  
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
- (B) STREET: 14 JOHN B. GORSIRAWEG
- (C) CITY: CURACAO
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- (F) POSTAL CODE (ZIP): NONE
- (G) TELEPHONE: 599-9-639300
- (H) TELEFAX: 599-9-614129

(ii) TITLE OF INVENTION: AMINO-TERMINALLY TRUNCATED RANTES AS  
CHEMOKINE ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 91 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION:1..68

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Leu Cys Ala Pro Ala Ser Ala Ser Pro Tyr Ser Ser Asp Thr Thr Pro  
          -5                          1                          5

Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys  
10                          15                          20                          25

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe  
                  30                          35                          40

09537858-032800

Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp  
45 50 55

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser  
60 65

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 66 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS:
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Leu Pro Arg Ala His Ile Lys Glu Tyr Phe Tyr Thr Ser Gly Lys Cys  
20 25 30

Ser Asn Pro Ala Val Val Phe Val Thr Arg Lys Asn Arg Gln Val Cys  
35 40 45

Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser Leu Glu  
50 55 60

Met Ser  
65

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